Mol Breeding (2008) 22:61–69
DOI 10.1007/s11032-008-9156-8

Development of a molecular marker associated with Verticillium wilt resistance in diploid interspecific potato hybrids

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Received: 27 June 2007 / Accepted: 28 December 2007 / Published online: 19 February 2008

Abstract Verticillium wilt (VW) is a widespread and serious potato (*Solanum tuberosum*) disease caused by the soilborne fungi *Verticillium dahliae* and *V. albo-atrum*. Breeding for VW resistance in potato is challenging due to ambiguous symptom expression, a lack of high throughput screening techniques, and variability in colonization by the fungus among and within plants. Genetic studies have identified major genes that confer resistance in diploid *Solanum chacoense* (V_c) and interspecific hybrids (V_w and V_t). However, to date, these genes have not been used to develop molecular markers for the identification of resistant clones. Tomato *Ve1* and *Ve2* gene sequence information was used to amplify candidate *Ve* gene orthologs from both resistant and susceptible diploid potato hybrids. A CAPS marker was generated to track VW resistance in a backcross population segregating for resistance. The marker was also tested for its usefulness in other breeding lines. Our results indicate that this marker is effective for selection of the V_w gene in segregating breeding populations.

Keywords Verticillium wilt · Interspecific potato hybrids · Disease resistance · CAPS marker · Dosage effect · *Ve* resistance gene

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Introduction

Verticillium wilt (VW) of potato (*Solanum tuberosum*), is an important soil-borne vascular disease. It is distributed throughout potato growing regions of the world and typically causes yield losses of 10–50% (Powelson and Rowe 1993). VW is caused by the fungi *Verticillium dahliae* Kleb, and *V. albo-atrum* Reink and Berth. Since the only effective control practice, soil fumigation, is expensive and has harmful environmental effects, host plant resistance has been explored as a method of disease control (Pegg 1974; Rowe et al. 1987). Most commercial cultivars in the United States are susceptible, although some exhibit moderate resistance (Rowe and Powelson 2002).

While functional VW resistance genes in potato have not been characterized, genes within the tomato *Ve* locus, conferring resistance to *V. dahliae*, have recently been isolated using positional cloning

(Kawchuk et al. 2001). Two closely linked genes (*Ve1* and *Ve2*) are encoded within the locus on the short arm of chromosome 9 and are likely the result of a recent duplication event (Kawchuk et al. 2001). Both *Ve* genes encode proteins with extracellular leucine-rich repeats and intracellular motifs that indicate a role in receptor-mediated endocytosis and protein-protein interactions (Kawchuk et al. 2001). When susceptible potato was transformed with either the tomato *Ve1* or *Ve2* gene, resistance was observed in the transgenic plants. This indicates a conservation of VW resistance signaling mechanisms between tomato and potato and, therefore, the possibility that orthologous *Ve* genes are present in some resistant potato lines.

To improve VW resistance in potato, it is important to identify new sources of resistance, investigate the genetic basis of resistance, and develop effective tools to screen segregating populations for resistant clones. VW resistance has been observed in several wild *Solanum* species (Concibido et al. 1994; Lynch et al. 1997; Jansky and Rouse 2000, 2003; Simko et al. 2004b). Lynch et al. (1997) reported that a single dominant gene in *S. chacoense* is responsible for resistance to *V. albo-atrum.* Jansky et al. (2004) identified two diploid interspecific hybrid clones (C287 and C545) with resistance to *V. dahliae* and have suggested that complementary gene action controls resistance.

The evaluation of potato breeding clones for VW resistance has been challenging because disease symptoms can easily be confused with natural senescence. Therefore, when the maturity of a line is not known in the breeding population, accurate disease diagnosis must be employed. Stem plating is currently the most common method for the quantification of stem colonization, but it is labor-intensive and time-consuming (Davis et al. 1983; Hoyos et al. 1993). Moreover, there are strong environmental influences on disease expression and high stem-to-stem variation for pathogen populations within and among plants (Frost et al. 2006, 2007). Because VW resistance is difficult to evaluate and major resistance genes have been identified, marker-assisted selection may provide a promising strategy to facilitate and accelerate the development of new VW resistant varieties (Simko et al. 2004c).

Simko et al. (2003, 2004a, b) used *Ve* gene information in tomato to detect *Ve* orthologs in tetraploid potato cultivars. VW resistance gene orthologs, showing high sequence identity with *Ve1* (83–90%) and *Ve2* (74–91%), were detected in the resistant cultivar 'Reddale'. Eleven *Ve1* orthologs, named *StVe1*, all mapped to the corresponding *Ve1* locus on chromosome 9, reflecting the complexity of the tetraploid potato genome. A marker was identified based on sequence variation in *StVe1*. However, the identified marker was linked to the susceptible allele at the locus (Simko et al. 2004b).

The objective of this study was to identify a molecular marker associated with VW resistance in diploid potato populations segregating for major resistance genes. A DNA marker was developed using sequence information from *Ve* orthologs in VW resistant diploid interspecific potato hybrids. The marker was then tested for association with resistance in segregating populations.

Materials and methods

Plant material

A cross was made between two diploid interspecific hybrid clones, C545 and C287, with high levels of resistance to VW (Jansky et al. 2004). The proposed genotypes of both resistant clones C545 and C287 are $V_wv_wV_tV_t$ or $V_wv_wV_tv_t$ where V_w and V_t correspond to alleles from wild *Solanum* species and *S. tuberosum*, respectively. In order to create a backcross population, a susceptible clone named V67, with a proposed genotype of $v_wv_wV_tv_t$, was identified from the cross between C545 and C287. V67 was then crossed to C545 to create a backcross population consisting of 105 clones.

True potato seeds were planted in a greenhouse on 20 April, 2005. On 14 May, 2005, seedlings were transplanted into pathogen-free Jiffy cell flats containing a commercial soil-less potting mix. In order to produce enough tubers for field evaluations, the 105 members of the backcross population were transplanted into 15 cm pots filled with potting mix on 14 June and three cuttings were made from each clone on 14 July. One month later, cuttings were transplanted into 15 cm pots and grown in a greenhouse. Tubers were harvested in September and stored in a cooler until the following spring. Tubers from 30 individuals were unsuitable for spring planting, reducing the population to 75 individuals.

Disease screening

Two two-hill replications of each of the 75 clones, along with the two parents, C545 and V67, were planted at the Hancock, Wisconsin, Agricultural Experiment Station on 2 May, 2006. Each plot was inoculated by spreading 30 g of dried, ground, *V. dahliae*-infested rye seeds into an open furrow at planting. The furrow was closed after the inoculum was spread. Clones were planted in a randomized complete block design with 30 cm within-row spacing and grown using best management practices for pest and disease control.

Soil samples were collected from 30 randomly chosen plots on 21 July. They were dried for 1 month at room temperature. For each soil sample, three 10 g subsamples were placed into a 250 ml flask to which 100 ml deionized water was added and stirred for at least 10 s. Two 1 ml aliquots from each flask were plated on nutrient pectate agar (NPX) as a selective medium (Butterfield and DeVay 1977) and spread evenly with a glass rod. After a 2-week incubation period in the dark, the number of *V. dahliae* colonies was counted on each plate using a dissecting microscope.

On 31 July, basal stem segments (approximately 10 cm in length) of all primary stems in each two-hill plot were collected and combined. Stems were surface sterilized with 1% NaOCl and rinsed in sterile distilled water. Sap was squeezed from each stem with a pair of pliers and a 200 µl aliquot was plated on a petri dish (10 cm) containing NPX medium. Following a 2-week incubation period at room temperature in the dark, the number of *V. dahliae* colonies in each petri dish was counted as a measure of stem colonization (Hoyos et al. 1991).

Ve gene ortholog amplification

Genomic DNA was extracted from young leaflets of resistant C545 and susceptible V67 plants using a DNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Primers were designed from the regions conserved (approximately 1,800 bp) between the two tomato *Ve* genes (*Ve1* and *Ve2*). Primers were designed using Vector NTI Suite software (Invitrogen, Carlsbad, CA). Synthesis of the primers was done by the UW-Madison Biotechnology Center. Primers C287F1 (5′-AACTGCAATTTCAGTGGAC-3′) and C287R1 (5′-TAACATAAAGTGAGCTAAGATCCC-3′) were used to amplify orthologous potato sequences from clones C545 and V67. The 50 µl PCR mixture consisted of 25 ng genomic DNA as a template, 1× PCR buffer, 0.25 U of Taq polymerase, 0.5 µM forward and reverse primers and 200 µM dNTPs. The thermal cycling conditions were 94° for 3 min followed by 35 cycles of 94° for 20 s, 55° for 40 s, 68° for 2 min conducted in a Bio-Rad iCycler (Hercules, CA). The amplified products were separated on a 1% (w/v) agarose gel run with 1× TAE buffer, stained with ethidium bromide and visualized under ultraviolet light. PCR products were gel purified using a Wizard® SV Gel Clean-Up System (Promega, Madison, WI) and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Ten plasmids containing inserts from both C545 and V67 PCR reactions were purified using a Wizard Plus SV Miniprep kit (Promega, Madison, WI). Positive and negative strands of the plasmid inserts were sequenced using vector (T7 and SP6) and internal primers using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with at least 2× coverage. Sequencing was performed at the UW-Madison Biotechnology Center using an ABI 3730xl DNA Analyzer. Sequences were analyzed using Vector NTI Suite software (Invitrogen, Carlsbad, CA).

Cleaved amplified polymorphic sequence (CAPS) marker development

Primer C287R2 (5′-CTAATGTTTTTAGAGCA-CAACC-3′) was designed using Vector NTI Suite (Invitrogen, Carlsbad, CA) in order to amplify the region surrounding the *Nco*I restriction endonuclease site. A CAPS marker was developed by digesting 5 µl of the C287F1/C287R2 PCR product with 5 units of the restriction endonuclease *Nco*I (New England Biolabs, Beverly, MA) and 1× NEB buffer 4 in a total volume of 20 µl. The reaction was incubated at 37°C for 5 h. Digested PCR products were resolved by electrophoresis in 3% (w/v) agarose gels in 1× TAE buffer stained with ethidium bromide.

Data analysis

The stem colonization data were analyzed using Proc GLM in SAS (SAS Institute Inc., Cary, NC). Means

were separated using the least significant difference (LSD) test at $P = 0.05$. The amplified DNA sequences from C545 and V67 were aligned with *Ve1* and *Ve2* in tomato using Clustal X, version 1.81 (Thompson et al. 1997). The data for the resistance phenotype and CAPS marker segregation ratios were tested for goodness-of-fit to predicted Mendelian inheritance ratios using the Chi-square test ($P = 0.05$).

CAPS marker verification

The CAPS marker was tested for the ability to detect VW resistance in VW resistant and susceptible breeding lines, the backcross population, and cultivars, to verify the usefulness of the marker. Tested plants were as follows: (1) 105 progeny from a cross between C545 and V67; (2) 11 offspring clones from the cross between interspecific diploid hybrid clones C287 and C545 (Jansky et al. 2004); (3) 30 diverse breeding lines with varying VW resistance phenotypes. The PCR amplification conditions and assays for the CAPS marker were the same as those described above.

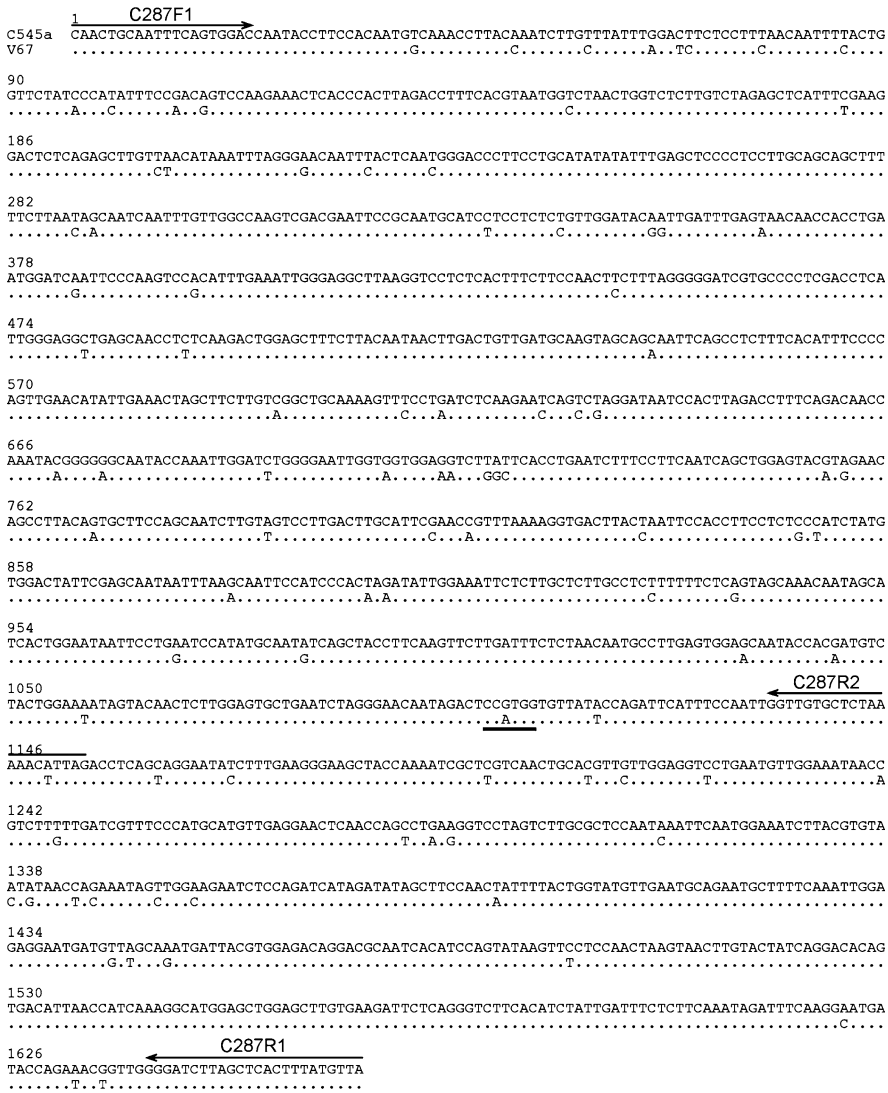
Results

We hypothesized that VW resistance in the diploid hybrid potato C545 was due to the presence of a functional ortholog of the tomato *Ve* resistance gene. Therefore, we developed *Ve*-specific primers for use in PCR with genomic DNA from resistant C545 and susceptible V67 hybrids. Amplification of genomic DNA from these parental clones using primer pairs C287F1 and C287R1 produced one 1,665 bp band. Cloning and subsequent sequencing revealed that the product derived from C545 contained a mixture of two different DNA fragments (named C545a and C545b), but the V67 template produced only one DNA fragment. The sequence of one of the DNA fragments in C545 (C545b) was the same as that of V67. There was a 5% sequence difference between C545a and C545b (Fig. 1). These results indicated that C545 is heterozygous and V67 is homozygous for the gene that was amplified. As expected, the amplified product shared significant (greater than 94%) sequence identity with the tomato *Ve2* gene.

The C545- and V67-derived sequences were compared in an attempt to develop a PCR-based marker capable of differentiating resistant and susceptible genotypes. The comparison revealed an *Nco*I restriction enzyme polymorphism between the C545a and V67 sequences. An *Nco*I restriction site is present within the V67 and C545b sequences, but is absent in C545a. Primer C287R2 was developed for amplification of the region surrounding the *Nco*I restriction site. Digestion of C287F1/R2 PCR products produced two fragments of the expected 1,154 and 50 bp sizes from V67 and three fragments (1,204, 1,154 and 50 bp) from C545 (Fig. 2a). The electrophoretic conditions necessary to adequately separate the 1,204 and 1,154 bp bands resulted in loss of the 50 bp band during analysis. Therefore, only one band for V67 and two bands for C545 were observed. We considered the 1,204 bp band to be a CAPS marker associated with VW resistance. When the CAPS marker was tested in an additional three clones, C287 and LTC 1821 (resistant *S. chacoense* clones) and V14 (susceptible C287 × C545 hybrid), the two resistant clones contained both the 1,204 and 1,154 bp bands, similar to C545. DNA from the susceptible clone V14 produced only the 1,154 bp band, which was the same as susceptible V67.

Correlation between the *Ve* CAPS marker and VW resistance in a segregating population

A marker association test was carried out with the diploid interspecific backcross population from a cross between resistant C545 and susceptible V67. Backcross seeds were produced when C545 was used as a female parent, but not as a male parent. Seventy-five progeny from this cross were planted in *Verticillium*-inoculated field plots. Field soil samples taken during the growing season contained 111 *V. dahliae* colony forming units (cfu) per gram. Although soil counts across replications ranged from 58 to 188 cfu/g, they were considerably higher than the threshold level for economic loss, which is approximately 10 cfu/g (Rowe et al. 1987). Mean stem colonization scores were used to quantitate VW resistance. Clones were considered to be resistant if they contained fewer *Verticillium* propagules than the susceptible parent V67, based on a LSD test. The mean stem colonization score of C545 was 55.75 ± 41.07 cfu/0.1 ml sap, which was



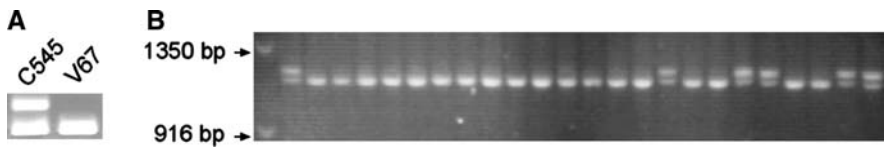


Fig. 1 Sequence alignment of *Ve*-like gene fragments from C545 and V67. The *Nco*I site (in V67 and C545b) used to develop the CAPS marker is underlined. Primers used for PCR amplifications are shown by arrows

Fig. 2 Genomic DNA from C545 (VW resistant) and V67 (susceptible) (**a**) and a representative example of progeny from a C545 × C287 cross (**b**) was amplified using primers C287F1 and C287R1. PCR products were digested using *Nco*I and separated on a 3% agarose gel for 8 h at 100 V

significantly different from susceptible V67 ($P = 0.05$), which had a mean stem colonization score of was 544 ± 379 cfu/0.1 ml. No significant differences were detected among replications for stem colonization scores.

Genomic DNA was extracted from the 75 field-grown C545 × V67 progeny. This genomic DNA was screened for the presence or absence of the CAPS marker associated with VW resistance (Fig. 2b). Since complementary gene action has been proposed as the

genetic basis for resistance in C545 (Jansky et al. 2004), resistant clones in this population were expected to contain dominant alleles for two genes. Our assumption was that the CAPS marker was associated with one of these genes. Based on this assumption, three possible observations were expected in the population: resistant phenotype and resistant marker, susceptible phenotype and susceptible marker, and susceptible phenotype and resistant marker. While these combinations were observed (Table 1), a fourth combination (resistant phenotype and susceptible marker) was observed in thirteen out of the 75 clones in the population. This result led us to consider the possibility of a dosage effect, where the presence of two dominant alleles from either or both of the resistance genes confers resistance. Using this assumption, the segregation ratio fit the model following a Chi-square analysis ($P = 0.18$, Table 1).

The VW resistance marker was tested to determine whether it could be used in other potato breeding clones with different genetic backgrounds. Two sets of clones were available. One was a population of 11 resistant clones derived from a cross between C545 and C287 (Jansky et al. 2004). All clones contained the resistant marker band, except for V65, which had only the 1,154 bp susceptible band. Two resistant clones, V25 and V34 showed only the 1,204 bp band, indicating that they might be homozygous for the resistance allele. The other group included 30 diverse clones, which varied for VW resistance (Table 2). The marker type predicted the phenotype in 25 of these clones.

Discussion

Marker assisted selection can be especially valuable for the early generation selection of VW resistant clones in potato. Qualitative resistance evaluation in the field is difficult due to ambiguous symptom expression and strong environmental influences on disease expression (Frost et al. 2006, 2007). Although major genes for VW resistance have been reported in *S. chacoense* and diploid interspecific hybrids (Lynch et al. 1997; Jansky et al. 2004), no molecular approach is available to identify these genes and accelerate resistance breeding progress. This study has developed a marker that detects VW resistance due to a major gene.

Only a few molecular markers for disease resistance in potato have been developed. These include markers for late blight resistance (Bisognin et al. 2005; Colton et al. 2006), resistance to *Potato virus Y* (Hamalainen et al. 1997, 1998; Kasai et al. 2000; Flis et al. 2005; Gebhardt and Bellin 2006; Witek et al. 2006) and resistance to the potato cyst nematode (Niewohner et al. 1995). Typically, markers have been developed using a genetic mapping approach. However this requires the development of a fine map in order to prevent recombination between the marker and the gene of interest.

The identification of orthologous *Ve* resistance genes in potato has been limited to cultivated tetraploid potato, *Solanum tuberosum* Tuberosum Group (Kawchuk et al. 2001; Simko et al. 2003, 2004a, b, c). However, inheritance of VW resistance in cultivated tetraploid potato appears to be polygenic and complex (Hunter et al. 1968; Pavek and Corsini 1994) and most cultivars are not highly resistant to VW (Rowe and Powelson 2002). A marker from an orthologous *Ve* gene in tetraploid potato was found to be linked to the susceptible allele (Simko et al. 2003, 2004b). While this marker could be useful in breeding efforts, marker-assisted selection would be more effective if a marker linked to a resistant allele could be identified. In this study, we chose to

Table 1 Association between stem colonization by *V. dahliae* and a CAPS marker in a backcross population derived from a cross between the diploid interspecific hybrid clones C545 ($V_w v_w V_t v_t$) and V67 ($v_w v_w V_t v_t$)

Disease reaction	Marker type	Observed	Expected	χ^2	*P*-value	cfu/0.1 ml	Postulated genotypes
R	R	24	28.125			41.5 ± 29.5	$V_w v_w V_t V_t$, $V_w v_w V_t v_t$
R	S	13	9.375			39.3 ± 28.9	$v_w v_w V_t V_t$
S	S	24	28.125			448.0 ± 291.4	$v_w v_w V_t v_t$, $v_w v_w v_t v_t$
S	R	14	9.375			466.6 ± 231.4	$V_w v_w v_t v_t$
	Total	75		4.89	0.18		

R, resistant; S, susceptible

Table 2 Association between stem colonization by *V. dahliae* and a CAPS marker in diverse breeding clones

Clone	Pedigree	Disease reaction	Marker type
C5	WisAg231 × (US-W2838 × *S. tarijense*) × C287	R	R
208329	Breeding stock	R	R
02-3056-7	cv. Montanosa × cv. Nevikij	R	R
598	Complex interspecific hybrid	R	S
593	Complex interspecific hybrid	R	R
671	Complex interspecific hybrid	R	R
501	Complex interspecific hybrid	R	R
519	Complex interspecific hybrid	R	R
745	Complex interspecific hybrid	R	S
933	Complex interspecific hybrid	MR	S
795	Complex interspecific hybrid	R	R
794	Complex interspecific hybrid	R	R
709	Complex interspecific hybrid	R	R
797	Complex interspecific hybrid	R	R
814	Complex interspecific hybrid	R	S
JP1	Somatically doubled C287	R	R
461	(US-W 13089 × *S. pinnatisectum* 275233) × *S. chacoense* 217451	S	S
3–7	*S. chacoense* 472819	R	R
4–1	*S. sparsipilum* 311000	R	R
4–2	*S. sparsipilum* 311000	R	R
4–5	*S. sparsipilum* 311000	R	R
4–6	*S. sparsipilum* 311000	R	R
+297	{[(*S. palustre* + PI203900 fusion) × cv. Katahdin] × cv. Atlantic} × cv. Atlantic	S	S
T450	(*S. palustre* + PI203900 fusion) × cv. Katahdin	S	S
Atlantic	Standard cultivar	MR	R
Red Norland	Standard cultivar	R	R
Superior	Standard cultivar	S	S
White Pearl	Standard cultivar	S	S
Russet Norkotah	Standard cultivar	S	R
Russet Burbank	Standard cultivar	MR	R

R, Resistant; MR, moderately resistant; S, susceptible

examine the relationship between tomato *Ve* genes and major dominant VW resistance genes in diploid potato clones (Lynch et al. 1997; Jansky et al. 2004).

Two diploid interspecific hybrid clones (C287 and C545) with resistance to *V. dahliae* are thought to contain complementary genes controlling VW resistance (Jansky et al. 2004). We first hypothesized that the genotypes of C545 and V67 were $V_wv_wV_tV_t$ and $v_wv_wV_tV_t$, respectively. The CAPS marker described here associates with only one of these genes, V_w. A 1:1 ratio of resistant to susceptible clones for VW resistance was observed in the backcross population. However, the marker did not co-segregate with VW resistance as expected. Based on the previous study by Jansky et al. (2004), a definitive genotype of the resistant clone C545 could not be interpreted, and could have been either $V_wv_wV_tV_t$ or $V_wv_wV_tv_t$ due to complementary gene action. According to the results of this study, we suggest that the genotype of C545 is $V_wv_wV_tv_t$. The genotype of the susceptible clone, V67, is $v_wv_wV_tv_t$. In the C545 × V67 population, we expected to observe resistant plants heterozygous for

the marker (1,204 and 1,154 bp bands), susceptible plants homozygous for the smaller band (1,154 bp), and susceptible plants ($V_w v_w v_t v_t$) heterozygous for the marker. However, we unexpectedly observed resistant plants lacking the resistance marker. The clones in this group may have the $v_w v_w V_t V_t$ genotype, with the V_t gene exhibiting a dosage effect. That is, two doses of V_t may confer resistance. Consequently, any genotype carrying two dominant V genes (V_w or V_t) would be resistant. Based on this assumption, the data fit the model (Chi-square analysis, $P = 0.18$, Table 1).

This marker was also successful in identifying other known resistant clones. The CAPS resistant marker (1,204 bp) was detected in *S. chacoense* clone LTC1821, which is reported to be heterozygous for a dominant VW resistance gene (Lynch et al. 1997). In addition, an analysis of 11 resistant clones selected from a cross between C545 and C287 revealed that all but one of the clones contain the resistant marker. With this marker test, we identified two clones homozygous for the resistant allele. In an assay of 30 widely diverse clones selected from other breeding populations, 25 clones had marker types that matched the phenotype (Table 2). Based on the marker test in these two different breeding populations, the presence of this newly developed CAPS marker segregates well with the resistance phenotype. However, in a few cases, plants that exhibited a resistance phenotype did not contain the resistant CAPS marker. We hypothesize that resistance in these clones is due to the presence of two V_t alleles or the presence of *Ve*-unrelated resistance genes.

The marker identified in this study will be useful for identifying resistant clones in populations segregating for one *Ve* ortholog. The PCR-based procedure used with this marker does not rely on expensive molecular methods and will be a valuable tool for potato breeders interested in selecting for VW resistance. Future research will be focused on identification of a marker linked to the V_t resistance gene in order to more accurately select resistant clones. We expect this strategy to accelerate progress for VW resistance breeding as major resistance genes are introgressed into populations.

Acknowledgments We would like to thank Sarah Stephenson and Andy Hamernik for expert technical assistance and critical review of the manuscript. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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